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Simultaneous determination of arsenic and selenium species in fish tissues using microwave-assisted enzymatic extraction and ion chromatography–inductively coupled plasma mass spectrometry

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ABSTRACT

A microwave-assisted enzymatic extraction (MAEE) method was developed for the simultaneous extraction of arsenic (As) and selenium (Se) species in fish tissues. The extraction efficiency of total As and Se and the stability of As and Se species were evaluated by analyzing DOLT-3 (dogfish liver). Enzymatic extraction using pronase E/lipase mixture assisted by microwave energy was found to give satisfactory extraction recoveries for As and Se without promoting interspecies conversion. The optimum extraction conditions were found to be 0.2 g of sample, 20 mg pronase E and 5 mg lipase in 10 mL of 50 mM phosphate buffer, pH 7.25 at 37 °C. The total extraction time was 30 min. The speciation analysis was performed by ion chromatography–inductively coupled plasma mass spectrometry (IC–ICP–MS). The accuracy of the developed extraction procedure was verified by analyzing two reference materials, DOLT-3 and BCR-627. The extraction recoveries in those reference materials ranged between 82 and 94% for As and 57 and 97% for Se. The accuracy of arsenic species measurement was tested by the analysis of BCR 627. The proposed method was applied to determine As and Se species in fish tissues purchased from a local fish market. Arsenobetaine (AsB) and selenomethionine (SeMet) were the major species detected in fish tissues. In the analyzed fish extracts, the sum of As species detected was in good agreement with the total As extracted. However, for Se, the sum of its species was lower than the total Se extracted, revealing the presence of Se-containing peptides or proteins.

1. Introduction

Arsenic (As) and selenium (Se) are characterized by their toxicological properties and widespread environmental occurrences. These metalloids are released into the environment through natural and anthropogenic sources, thus exposing humans to increased concentrations of both elements in food, water and other environmental media [1,2]. With respect to As, fish and shellfish diets account for the majority of ingested As, where the total As concentration ranged from 1 to 100 mg kg^{-1}. Due to the variable levels of toxicity associated with As species in foods, knowledge of the actual forms present, rather than only the total As, is necessary in order to assess potential harmful contamination [3]. Inorganic As species, such as As(III) and As(V), have been classified as carcinogens and methylated forms, such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), have been labeled as cancer promoters. Arsenobetaine (AsB), the predominant As species in fish and shellfish, is believed to have negligible toxicity [1,3,4]. Regarding Se, its speciation is of particular interest since it is recognized as both an essential nutrient and a toxin to mammals, and the concentration interval range between essentiality and toxicity is very narrow and strongly dependent on its chemical form [1,2]. Inorganic forms of Se are generally more toxic than organic Se compounds, and inorganic Se(IV) is the most toxic species [2]. Most of the Se in mussel and fish tissues has been associated with organic forms such as selenomethionine (SeMet) [5]. Trimethylselenonium ($TMSe^+$) was detected in oyster, mussel and trout, while inorganic Se was found in krill [6]. Although seafood is a significant source of Se, in some cases fish tissue is a poor source of available Se due to the interaction of Se with a number of toxic metals, such as As, which binds to Se forming insoluble inorganic complexes [7,8]. Antagonistic effects between As and Se have been confirmed in many animal species, including humans [9]. Techniques for the simultaneous speciation of As and Se are important to support these findings.

Currently, liquid chromatography (LC) paired with inductively coupled plasma mass spectrometry (ICP–MS) for element selective detection has been predominantly applied for As and/or Se spe-

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ciation. Ion-exchange has been widely used for separating As or Se compounds in biological materials [6,10–13]. However, a limited number of separation systems for the simultaneous speciation of As and Se in biological samples have been reported. Recently, Wang et al. [14] developed an anion-exchange separation of As(III), As(V), MMA, DMA, AsB, Se(IV) and Se(VI), and the method was applied to the analysis of urine samples and fish extracts.

Sample preparation is the most important and limiting step for speciation analysis in solid dietary matrices. Extraction conditions must be chemically mild but efficient to quantitatively extract analytes from different matrices while maintaining the integrity and the concentration of individual species. Different water/methanol mixtures [15–17] have been commonly used for the extraction of As species from fish tissues. It has been reported, however, that incomplete extraction of inorganic As from biological matrices occurs due to the inability of water/methanol mixtures to break the bonds between As(III) and thiol groups (–SH) in proteins [12]. Alternative methods based on solubilization with tetramethyl ammonium hydroxide (TMAH) for the extraction of As species from marine tissues have been described in the literature [12,18]. Enzymatic extractions with trypsin have also been investigated for As speciation in fish and crustacean samples [19]. Conversely, common extraction methodologies for Se speciation include enzymatic and alkaline extractions, the former being the most widely used. Several enzymes, including protease XIV [20], pronase E [6,10,21], pronase E/lipase mixture [22,23] and trypsin [21], have been applied to Se speciation studies in various biological tissues. Generally, the use of enzymatic hydrolysis has shown better results in the release of Se species from biological solid samples than basic extraction conditions (using TMAH reagent) that lead to Se species degradation [5]. Among the procedures reported in the literature for the extraction of As or Se, enzymatic extraction is of interest due to the use of moderate temperature and pH conditions that prevent elemental losses by volatilization and minimize species degradation. However, the main drawback of enzymatic hydrolysis is the significant time required to complete the hydrolysis process (12–24 h).

In recent years, the use of microwave-assisted extraction (MAE) has gained wide acceptance as a powerful tool for sample preparation of solid matrices for speciation analysis. In contrast with traditional extraction techniques such as mechanical shaking or sonication, sample preparation time and solvent amount are reduced without decreasing the extraction efficiency of the target species [24].

The objective of this study was to evaluate the feasibility of microwave-assisted enzymatic extraction (MAEE) for the simultaneous extraction of As and Se species from fish tissues as an alternative to conventional enzymatic extraction procedures. The combined use of enzymes and closed-vessel microwave-assisted extraction for the multielemental species extraction has not been previously reported. A critical study of the most important parameters involved in the extraction procedure, followed by the comparison of several common extraction procedures used for As or Se in fish tissues, was performed. As and Se species were separated using an anion-exchange chromatography and detected on-line with an inductively coupled plasma mass spectrometer. The results are presented in this article.

2. Experimental

2.1. Instrumentation

The chromatography system (Metrohm-Peak, LLC, Houston, TX, USA) consisted of two 818 IC Pumps, a 762 IC Interface, and an 838 IC Autosampler. The separation center enclosure included a six-port sample injector equipped with a 100 μL sample loop. Ion-exchange chromatography was performed in a Metrosep Anion Dual 3 analytical column with a Metrosep Anion Dual 3 guard column (Metrohm-Peak, LLC, Houston, TX, USA). A gradient elution of 5 mM and 50 mM NH_4NO_3, 2% (v/v) MeOH (pH 8.7) was used. Size exclusion chromatography (SEC) was performed on a TSK–gel QC-PAK GFC 200 column (Tosoh Bioscience LLC, Montgomeryville, PA, USA) with a molecular weight exclusion limit of 150 kDa. The chromatographic system was coupled directly to the nebulizer of the ICP–MS by a small piece of perfluoroalkoxy (PFA) tubing.

Table 1
ICP–MS instrumental settings and chromatographic separation conditions.

IC	
Column	Metrosep™ Anion Dual 3 column (100 mm × 4.0 mm, 6 μm) and Metrosep™ Anion Dual 3 guard column (1.7 mm × 3.5 mm, 0.2 μm) (Metrohm Peak, LLC.)
Mobile phase	A: 5 mM NH_4NO_3, and B: 50 mM NH_4NO_3, 2% (v/v) methanol, pH 8.7
Gradient program	1: 0 min, 0% B, 2: 2 min, 0% B, 3: 7 min, 100% B, 4: 9 min, 100% B, 5: 9.5 min, 0% B and 6: 12 min, 0% B.
Flow rate	1 mL min^{-1}
Injection volume	100 μL
Column temperature	Ambient
SEC	
Column	TSK–gel QC-PAK GFC (200 column, 150 mm × 7.8 mm, 5 μm) (Tosoh Bioscience)
Mobile phase	50 mM phosphate buffer, 2% (v/v) methanol, pH 7.25
Flow rate	0.7 mL min^{-1}
Injection volume	100 μL
Column temperature	Ambient
ICP–MS	
Rf power	1475 W
Plasma argon flow	15.0 L min^{-1}
Auxiliary argon flow	1.0 L min^{-1}
Monitoring isotopes	^{75}As[a,b], ^{77}Se[a,b] and ^{82}Se[a,b]
Acquisition mode	Spectrum[a] and time-resolved analysis[b]
Integration time per mass (s)	0.30[a] and 0.20[b]
Replicates	5[a] and 1[b]
Total analysis time (s)	34.45[a] and 718[b]

[a] For total As and Se analysis.
[b] For As and Se speciation analysis.

The ICP–MS instrument was an Agilent HP-4500 (Palo Alto, CA and Yokogawa Analytical Systems Inc., Tokyo, Japan). The sample introduction system consisted of a concentric nebulizer with a Scott double-pass quartz spray chamber. The operating parameters are summarized in Table 1. The torch position and ion lens voltage settings were optimized daily for optimum sensitivity with a standard solution of 10 μg L^{-1} Li, Y, Ce, and Tl in 2% (v/v) HNO_3. Ion intensities at m/z 75, 77 and 82 were recorded using spectrum mode for total analysis and time-resolved analysis (TRA) for speciation analysis.

An ETHOS 1 laboratory microwave system [Milestone, Sorisole (BG), Italy] equipped with temperature and pressure feedback controls, magnetic stirring capability and 10 high pressure vessels of 100 mL inner volume, operating at a maximum exit power of 1500 W, was employed for the digestion and extraction processes.

The commercial fish tissues were freeze–dried for 48 h (Freezone 4.5, Labconco Inc., Kansas City, MO). A FAM-40 vacuum unit (Milestone, Sorisole, Italy) was used to filter digest and extracts. A centrifuge (Model 225, Fisher Scientific Co., St. Louis, MO, USA) was employed for the sample preparation.

2.2. Reagents and materials

All reagents were of analytical reagent grade. Double-deionized (DDI) water with a resistivity of 18 MΩ-cm obtained from a Barnstead NANOpure Water System (Dubuque, IA, USA) was used

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throughout. Hydrogen peroxide 35% (w/v) in water and ammonium phosphate dibasic were purchased from Acros Organic (New Jersey, USA). TMAH, nitric acid, ammonium bicarbonate and optima grade methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Ammonium phosphate monobasic, ammonium nitrate, ammonium hydroxide solution (20–22% NH_3), trypsine from bovine pancreas, pronase E from *Streptomyces griseus* and lipase VII from *Candida rugosa* were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

Stock standard solutions for As were: As(III) and As(V), 1000 mg As L^{-1} from SPEX CertiPrep (Metuchen, NJ, USA); MMA, DMA, and AsB (1000 mg As L^{-1}) were prepared by dissolving the appropriate amounts of sodium methylarsonate [$CH_3AsO(ONa_2)$•$6H_2O$] from ChemService (West Chester, PA, USA); sodium cacodylate [$C_2H_6AsNaO_2$•$3H_2O$] from Sigma (St Louis, MO, USA); and AsB ($AsC_5H_{11}O_2$) from Argus Chemicals (Florence, Italy), in DDI water. Stock standard solutions of Se(IV), Se(VI) and SeMet (1000 mg Se L^{-1}) were prepared by dissolving adequate amounts of sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4) and SeMet ($C_5H_{11}NO_2Se$), from Sigma (St Louis, MO, USA) in DDI water. The standard solutions were stored in amber high density polypropylene containers at 4 °C in a cold room. Analytical working standards were prepared daily by diluting the stock solutions with DDI water prior to analysis.

The following samples were used in this study: certified reference materials DOLT-3 Dogfish Liver [National Research Council of Canada (NRCC), Ontario, Canada] and BCR-627 Tuna Fish Tissue [Institute for Reference Materials and Measurements (IRMM), Geel, Belgium] together with fish tissues purchased at Pittsburgh, PA fish markets (shark, canned tuna in water, and marlin). The certified reference materials were used as received; all other samples were freeze–dried prior to the extraction procedure.

2.3. Procedures

2.3.1. Microwave-assisted digestion procedure

For the analysis of total As and Se, the samples were digested using EPA Method 3052 [25]. Approximately 500 mg representative samples were weighed into microwave vessels; 9 mL concentrated HNO_3 and 0.5 mL H_2O_2 along with magnetic stir bar were added into each vessel. Vessels were then sealed and microwave-irradiated at 180 ± 5 °C for 10 min. After digestion, the digests were filtered through 0.22 μm Millipore glass fiber filters (Fisher Scientific, Pittsburgh, PA, USA). The digests were diluted to 30 mL with DDI water and stored in a cold room at 4 °C until analysis (usually within 48 h).

2.3.2. MAEE procedure

A 200 mg portion of sample with 20 mg of pronase E and 5 mg of lipase was weighed into individual Teflon digestion vessel; 10 mL of 50 mM phosphate buffer (pH 7.25) were added and a magnetic stir bar was placed into each vessel for thorough mixing of solvent with the sample. The microwave vessels were sealed and irradiated at 37 °C for 30 min and the ramp time was set as 10 min. After microwave heating, the vessels were allowed to cool to room temperature and the extracts were centrifuged for 10 min at 4000 rpm. The supernatants were filtered through 0.22 μm glass fiber filters followed by a 1 + 3 fold dilution with DDI water. The extracts were stored at 4 °C in a cold room for analysis within 48 h. Blanks were prepared along with the samples in each batch.

2.3.3. As and Se species stability

A 200 mg of DOLT-3 was spiked with 100 μL of individual As and Se species [As(III), As(V), MMA, DMA, AsB, Se(IV), Se(VI) and SeMet] standard solution of 10 mg L^{-1}, to which the extraction solvent was added. Samples were left for 1 h to equilibrate at room temperature, and then 20 mg of pronase E, 5 mg of lipase and 10 mL of 50 mM phosphate buffer (pH 7.25) were added. Three replicates were prepared in each case. The microwave vessels were sealed and irradiated at 37 °C for 30 min. Finally, the extracts were analyzed by IC–ICP–MS in the same way as the samples to verify As and Se species stabilities during the extraction procedure.

2.3.4. Analysis by ICP–MS and IC–ICP–MS

All samples were further diluted and analyzed by ICP–MS and IC–ICP–MS.

2.3.4.1. Total As and Se determination by ICP–MS. Total As and Se concentrations in the extracts and digests were determined by ICP–MS in spectrum mode. The isotopes ^{75}As, ^{77}Se and ^{82}Se were monitored. Samples were quantified by external calibration using matrix matching. Calibration was performed by using 0.5–20 μg L^{-1} mixture of inorganic As and Se standards in 0.5% (v/v) HNO_3. The operating conditions are given in Table 1. For Se, the Se isotope m/z 82 was used for data evaluation; similar result was obtained using the Se isotope m/z 77.

2.3.4.2. Determination of As and Se species. The extracts for As and Se species were analyzed by IC–ICP–MS and SEC–ICP–MS. For ICP–MS data acquisition, the time-resolved analysis mode was used. Each sample was analyzed three times. As and Se compounds were quantified by external calibration. Data evaluation was performed using the ChemStation software supplied with the instrument; quantification was performed based on peak areas.

3. Results and discussion

3.1. Determination of total As and Se in fish tissues

Total As and Se concentrations in DOLT-3, BCR-627 and fish tissues were determined after microwave acid digestion (EPA Method 3052) [25] by ICP–MS under conditions given in Table 1. The analytical results are shown in Table 2 (n = 3). The results for As and Se in DOLT-3 and BCR-627 using the proposed digestion procedure were in good agreement with the certified values at 95% confidence level. The content of As in the analyzed fish tissues ranged from 1.11 to 27 mg kg^{-1}. The values of As in these fish samples were comparable with other data found in fish [26,27]. Se concentrations in fish

Table 2
Total As and Se concentrations (mg kg^{-1}) found in fish tissues and the certified reference materials by ICP–MS after microwave digestion (EPA Method 3052).

Sample	Total As (mg kg^{-1})		Total Se (mg kg^{-1})	
	Certified value	Concentration found[a]	Certified value	Concentration found[a]
BCR-627 (IRMM, Belgium)	4.8 ± 0.3	4.8 ± 0.1	-	2.8 ± 0.2
DOLT-3 (NRCC, Canada)	10.2 ± 0.5	10.0 ± 0.2	7.06 ± 0.48	7.6 ± 0.2
Canned tuna (in water)		2.9 ± 0.1		5.6 ± 0.2
Shark		27 ± 1		2.0 ± 0.1
Marlin		1.11 ± 0.04		3.9 ± 0.1

[a] The results are expressed as mean value at 95% CL (n = 3).

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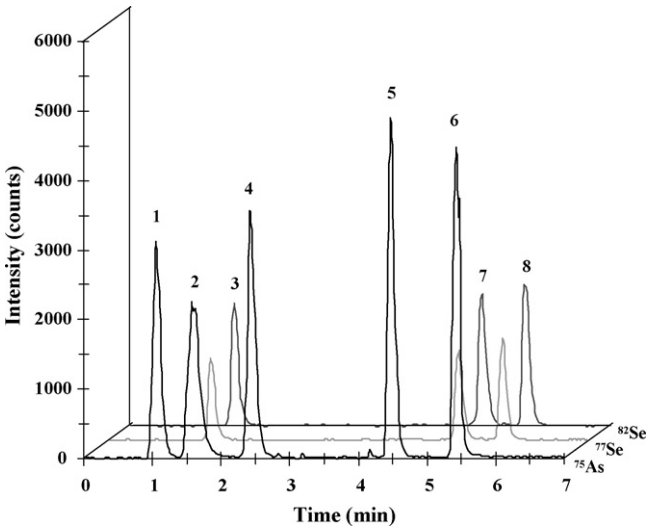

Fig. 1. IC–ICP–MS chromatogram of a 100 μL injected standard solution containing 2 μg L^{-1} of each As species and 8 μg L^{-1} of each Se species. Peak identification: (1) AsBet, (2) As(III), (3) SeMet, (4) DMA, (5) MMA, (6) As(V), (7) Se(IV), and (8) Se(VI). The analysis was performed according to the optimum conditions shown in Table 1.

tissues varied between 2 mg kg^{-1} (shark) and 5.6 mg kg^{-1} (canned tuna). Se levels were higher than those published in the literature. The reported values were in the range of 0.25–1.02 mg kg^{-1} [5,27,28]. Such differences could indicate bioaccumulation of Se in the analyzed fish samples.

3.2. Ion chromatography separation.

In this study, anion-exchange chromatography was employed for the separation of five As species and three Se species. Ammonium nitrate (NH_4NO_3) was selected as the mobile phase with concentrations ranging from 5 to 50 mM at pH 8.7 based on a better sensitivity of the species of interest and lower retention times. Additionally, the use of NH_4NO_3 as mobile phase has been shown to produce good signal stability on the ICP–MS with minimal salt deposit on the sample and skimmer cones [29]. The ionization of As and Se in the plasma was improved by adding 2% (v/v) methanol to the mobile phase. The chromatographic conditions are listed in Table 1. Fig. 1 shows an IC–ICP–MS chromatogram obtained for a standard solution containing 2.0 μg L^{-1} of As per species [AsB, As(III), DMA, MMA, and As(V)] and 8.0 μg L^{-1} of Se per species [SeMet, Se(IV), and Se(VI)]. Separation of As and Se species was resolved to baseline in less than 7 min. All calibrations were linear for As and Se species over the concentration range of 0.5–40 μg L^{-1} and 1.5–20 μg L^{-1}, respectively. Detection limits (S/N = 3) with a 100 μL of injection volume were about 0.1 μg L^{-1} for As species and 0.7 μg L^{-1} for Se species. The reproducibility from multiple injections (n = 5) containing a mixture of As and Se species standard solution of 2.0 μg L^{-1} and 8 μg L^{-1}, respectively, showed that the RSD were less than 5% for all the species investigated.

3.3. Optimization of the MAEE procedure of As and Se species

Methods based on enzymatic hydrolysis have been widely used to efficiently extract protein-bound Se species [6,10,21,23] and arsenocompounds [19] in various fish tissues. In this study, the combination of pronase E/lipase mixture and MAE was evaluated for the first time for the simultaneous extraction of As and Se species in fish tissues. Preliminary experiments were carried out to develop the appropriate extraction conditions. The following variables were optimized in DOLT-3 certified reference material: amount of pronase E and lipase, extraction pH, and irradiation time (Fig. 2). All experiments were carried out in triplicate with 200 mg of a lyophilized reference material. Irradiation temperature was maintained at 37 °C during the extraction (based on information from enzyme supplier). The minimum amount of solvent recommended by the manufacturer of the microwave system, 10 mL, was used in this study. Extraction efficiencies were calculated based on the certified values for As and Se in DOLT-3 sample.

3.3.1. Amount of pronase E

The mass of pronase E was varied from 10, 20 or 40 mg (corresponding to 5:1, 10:1 and 20:1 sample/enzyme mass ratio), and a 10 mg portion of lipase in 10 mL phosphate buffer (pH 7.5) was added to the DOLT-3 reference material. Solutions were irradiated in a closed-vessel microwave oven at 37 °C for 1 h. As can be seen in Fig. 2a, the percent recovery of As increased from 74 ± 1% when the extraction was completed with no pronase E, to 85 ± 1%, in the presence of 40 mg of enzyme. As previously described [30], these results could indicate that As in fish tissue is not linked to proteins. With respect to Se, the amount of pronase E had a pronounced affect on its extraction efficiency. The percent recovery was found to increase from 26 ± 1% without pronase E, to 86 ± 3% using 40 mg of pronase E. Evidently, a high fraction of total Se in fish tissues is associated with proteins. Since there is no statistically significant difference between the extraction recovery of As and Se using either 20 or 40 mg of pronase E, 20 mg of pronase E (10:1 sample/enzyme mass ratio) was chosen for further experiments to minimize reagent consumption, and reduce the cost of the experiment. The sample/enzyme mass ratio of 10:1 has been widely used for the extraction of Se species in different biological materials, such as selenized yeast and fish tissues [6,20,22].

3.3.2. Amount of lipase

Enzymatic extraction using pronase E/lipase mixture has been reported to be the most efficient approach for quantitative extraction of Se in biological samples [22,23]. In order to evaluate the effect of lipase on the extraction of As and Se from DOLT-3 reference material, a 200 mg of sample was treated with 20 mg pronase E and varying amounts of lipase (from 0 to 20 mg). The microwave extraction was performed at 37 °C for 1 h. Results are shown in Fig. 2b. The amount of lipase did not significantly affect the extraction efficiency of As and Se in DOLT-3. A slight increase of the extraction efficiency for both As and Se was observed using 5 mg lipase. Although 20:1 (sample/lipase mass ratio) has been commonly reported for the extraction of Se compounds in biological matrices [22,23], a smaller amount of lipase (sample/lipase mass ratio of 40:1) was required in this study for the simultaneous extraction of As and Se in fish tissues. The percent recovery of As and Se at the aforementioned sample/lipase ratio was 84 ± 1 and 90 ± 3%, respectively.

3.3.3. pH

Typically, enzymatic extraction is carried out in a buffer solution (Tris–HCl or phosphate buffer) under the standard conditions of pH that are recommended by the manufacturer of selected enzymes (pH 7.2 for lipase and 7.5 for pronase E). The application of a buffer solution is recommended in some matrices since the enzymes have optimal pH ranges, rendering necessary careful control of pH when the enzymatic procedure is in progress [31]. The pH range between 7.0 and 7.5 using 50 mM phosphate buffer was evaluated (see Fig. 2c). A modest improvement in the extraction efficiency of As and Se was observed when the pH was ≥7.25. The extraction recovery for As and Se was 80 ± 1 and 85 ± 3%, respectively, by using pH 7.25. A pH value of 7.25 was selected to comprise a satisfactory extraction recovery for As and Se.

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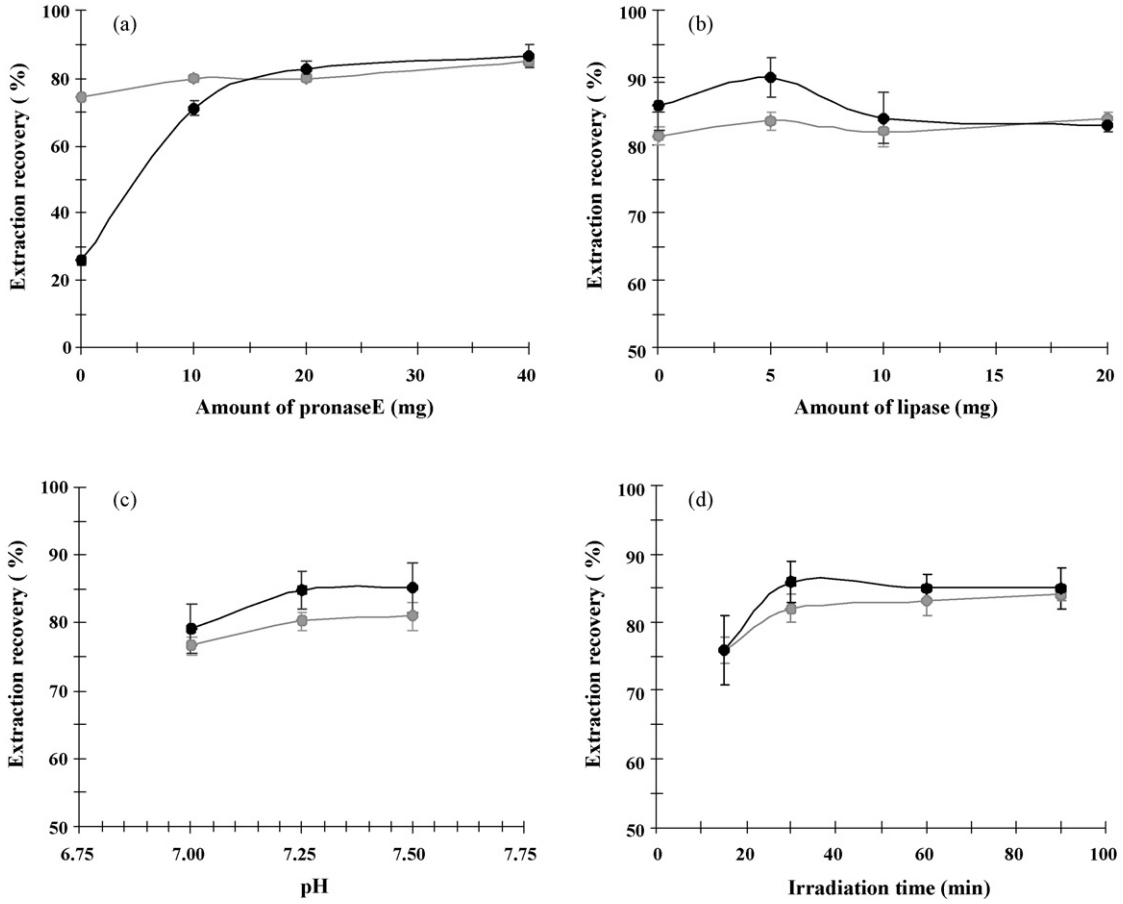

Fig. 2. Recovery of As (●) and Se (–●–) from DOLT-3 dogfish liver certified reference material during the evaluation of the following MAEE parameters: (a) amount of pronase E, (b) amount of lipase, (c) pH and (d) irradiation time. Results were expressed as the mean value ± standard deviation ($n = 3$).

3.3.4. Irradiation time

Irradiation time ranging from 20 to 90 min was investigated at 37 °C. As can be seen from Fig. 2d, a slight increase in the extraction efficiencies of both As and Se was observed for times longer than 30 min. At 30 min irradiation time, the extraction recoveries of As and Se were 83 ± 2 and 85 ± 3%, respectively. Since longer extraction times did not further improve percent recovery of As and Se from DOLT-3, an extraction time of 30 min was selected as the optimal time throughout the rest of the study.

The following extraction conditions were adopted for the simultaneous extraction of As and Se in fish tissues: 20 mg pronase E, 5 mg lipase, 10 mL of 50 mM phosphate buffer (pH 7.25) and an extraction time of 30 min. The advantage of this extraction procedure is the shorter extraction time, compared to traditional enzymatic approaches that normally require a treatment period of 20 h. Additionally, the proposed MAEE extraction method offers significant advantages include multielemental speciation extraction capabilities, high sample throughput, adequate temperature control, and minimum risk of contamination or loss of the analyte since the reaction takes place in a closed system.

3.3.5. Stability of As and Se species during MAEE

The stability of the As and Se species under the extraction conditions was also determined by spiking individual As [As(III), DMA, MMA, As(V) and AsB] and Se species [Se(IV), Se(VI) and SeMet] into the dogfish liver reference material. Single peaks were identified for all the species following IC–ICP–MS analysis with extraction recoveries in the range 99–106% for As, and 97–104% for Se species of the original spiked standard solutions. The results confirmed the absence of significant losses or transformations of As and Se species during the MAEE procedure.

3.4. Comparison of the developed method with common extraction procedures

The capability of the proposed method for the simultaneous extraction of As and Se was compared with common extraction procedures used for the extraction of As [16,18,19] or Se [6,10,22] in DOLT-3 reference material. The extraction recovery was then calculated based on the certified values of total As and Se in DOLT-3. The comparative results are shown in Table 3. The evaluated extraction procedures were based on the application of the following reagents: 80% (v/v) methanol in water, 8.3% (v/v) TMAH in water and three different enzymes: trypsin, pronase E and pronase E/lipase. The evaluated enzymatic digestion procedures were performed with incubation in a water bath at 37 °C for 12 h (trypsin procedure) and 20 h (pronase E and pronase E/lipase procedures) and with microwave irradiation at 37 °C for 30 min (pronase E/lipase).

Results for total As determination in DOLT-3 (Table 3) showed that the lowest extraction recovery (63% from the certified value) was obtained with aqueous 80% (v/v) MeOH. Lower extraction recoveries of As (76%) have also been reported in dogfish liver reference material (DOLT-1) using 50% (v/v) methanol in water extraction [15]. The conventional enzymatic extraction procedures using trypsin, pronase E, pronase E/lipase provided extraction recoveries between 73 and 76%. The differences in the extracted As were not statistically significant among the three enzymes (one-way ANOVA test, $P < 0.05$) when conventional enzymatic extraction procedures was used. Regarding the enzymatic extraction with the

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Table 3
Total As and Se concentration found in DOLT-3 CRM (dogfish liver) following different extraction procedures ($n = 3$, at 95% CL).[a].

Extraction reagent	Extraction conditions	Concentration found (mg kg^{-1})[b]		Ref.
		As	Se	
80% (v/v) methanol in water	200 mg of sample and 10 mL of 80% (v/v) methanol in water. MAE at 80 °C for 5 min	6.4 ± 0.1 (63 ± 1)	0.94 ± 0.05 (13 ± 1)	[18]
8.3% (v/v) TMAH in water	300 mg of sample and 9 mL of the mixture containing 3 mL 25% (w/v) TMAH solution and 6 mL DDI water. MAE at 50 °C for 20 min	9.0 ± 0.1 (88 ± 1)	7.6 ± 0.2 (107 ± 2)	[21]
Trypsin	250 mg of sample, 100 mg trypsin and 20 mL 0.1 M carbonate buffer (pH 8). Conventional enzymatic extraction at 37 °C for 12 h	7.7 ± 0.2 (76 ± 2)	5.0 ± 0.1 (70 ± 2)	[22]
Pronase E	200 mg of sample, 20 mg pronase E and 10 mL 50 mM phosphate buffer (pH 7.5). Conventional enzymatic extraction at 37 °C for 20 h	7.6 ± 0.1 (74 ± 1)	5.0 ± 0.3 (71 ± 4)	[6]
Pronase E/lipase	200 mg of sample, 20 mg pronase E, 10 mg lipase and 10 mL 50 mM phosphate buffer (pH 7.5). Conventional enzymatic extraction at 37 °C for 20 h	7.4 ± 0.2 (73 ± 3)	5.4 ± 0.1 (77 ± 1)	[25]
Pronase E/lipase	200 mg of sample, 20 mg pronase E, 5 mg lipase and 10 mL 50 mM phosphate buffer (pH 7.25). MAE at 37 °C for 30 min	8.4 ± 0.1 (82 ± 1)	5.9 ± 0.2 (84 ± 3)	This study

[a] Certified values for total As and Se in DOLT-3 CRM are 10.2 ± 0.5 and 7.06 ± 0.48 mg kg^{-1}, respectively.
[b] In parentheses, the percent recovery of different extraction methods (%) referred to the certified value.

proteolytic enzyme pronase E, the extraction recovery of As was not enhanced using pronase E/lipase mixture. However, higher extraction recovery for As was obtained using this mixture of enzymes when the extraction procedure involved the action of microwave compared with the conventional enzymatic procedure. The results showed that the extraction recovery of As in DOLT-3 was 82 ± 1%. It is observed from Table 3 that the TMAH procedure using MAE provided the highest extraction recoveries for As (88 ± 1%), although interconversion of As(III) to As(V) was observed during the extraction with TMAH. Results obtained during the current study were in agreement with those from previous studies reported in the literature [12].

With respect to Se, extraction with methanol/water mixture provided the lowest amount of leached Se from DOLT-3 (13%). Similar results were reported by Casiot et al. [22] in selenized yeast using water/methanol extractant. The results for the three enzymatic procedures using incubation in water bath at 37 °C showed statistically significant differences in the extraction recovery of Se (one-way ANOVA test, $P > 0.05$). The extraction recoveries of Se were in the range from 70% (trypsin) to 77% (pronase E/lipase). It is known that trypsin is a specific proteolytic enzyme that breaks peptide bonds at specific regions of the protein chain, while pronase, an unusually non-specific proteolytic enzyme, can act along the entire protein structure [31]. These differences in the mechanism of the two enzymes may explain the higher extraction efficiency of pronase compared to that of trypsin (see Table 3). On the other hand, an increase in the extraction yield was obtained using pronase E/lipase mixture compared with pronase probably due to the significant amount of Se bound to lipids indicating that the use of lipase is justified in this matrix. The application of microwave technology for the pronase E/lipase mixture increased the extraction recovery of Se from DOLT-3 to 84 ± 3%, compared to 77 ± 1% for the conventional enzymatic procedure using pronase/lipase. The microwave technique appears to provide effective disruption of the sample, thus facilitating enzyme interaction with liberated compounds, resulting in a reduction of sample treatment time from 20 h to 30 min. Although quantitative Se extraction recoveries were achieved using TMAH reagent (107 ± 2%), Se species present in the sample were degraded to inorganic Se during extraction. Conversion of original Se species to Se(VI) in selenized yeast has been reported when alkaline hydrolysis was performed using 25% (v/v) TMAH reagent [22]. Results from this comparative study demonstrate that MAEE procedure using pronase E/lipase mixture is the most suitable method for the simultaneous extraction of As and Se in fish tissues.

3.5. Determination of total As and As species in fish tissue extracts after MAEE procedure

The robustness of the optimized extraction MAEE procedure was assessed using two marine certified reference materials (BCR-627 and DOLT-3) and three fish tissues. Following enzymatic extraction, the samples were analyzed for total As by direct ICP–MS and for As species applying the above optimized IC–ICP–MS method. The results are shown in Table 4. For the reference materials, the extraction recoveries of total As were 94 ± 3%, and 82 ± 1% for BCR-627 and DOLT-3. Lower extraction recoveries of total As have been previously published in dogfish liver reference material. Kirby et al. [15] reported an extraction efficiency of 62% in DOLT-1 after water/methanol extraction, and Wahlen et al. [13] reported an extraction efficiency of 55% using water/sonication protocol. The

Table 4
Analytical results for total As and As species after the proposed MAEE procedure. The results are indicated at 95% CL for $n = 3$.

Sample	Total As extracted (mg kg^{-1})	As containing species (mg kg^{-1})					Sum of the species (mg As kg^{-1})[c]
		AsB	As(III)	DMA	MMA	As(V)	
BCR-627 (IRMM, Belgium)[d]	4.5 ± 0.1 (94 ± 3)[a]	4.17 ± 0.07	ND[e]	0.18 ± 0.01	ND[e]	ND[e]	4.4 ± 0.1 (98 ± 2)
DOLT-3 (NRCC, Canada)	8.4 ± 0.1 (82 ± 1)[a]	7.2 ± 0.1	ND[e]	0.48 ± 0.01	ND[e]	0.08 ± 0.02	7.7 ± 0.1 (92 ± 1)
Canned tuna (in water)	3.1 ± 0.1 (106 ± 2)[b]	2.8 ± 0.2	0.04 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	<LOD[f]	2.9 ± 0.2 (94 ± 6)
Shark	27.4 ± 0.8 (101 ± 3)[b]	25.7 ± 0.4	2.7 ± 0.1	0.03 ± 0.01	ND[e]	0.06 ± 0.01	28.4 ± 0.5 (103 ± 2)
Marlin	0.70 ± 0.01 (63 ± 1)[b]	0.72 ± 0.07	<LOD[f]	ND[e]	ND[e]	0.04 ± 0.01	0.76 ± 0.07 (109 ± 10)

[a] In parentheses, the extraction recovery based on total As certified values.
[b] In parentheses, the extraction recovery based on the total As determined by microwave-assisted total digestion (EPA Method 3052).
[c] In parentheses, total As speciation recovery based on total As extracted.
[d] Certified values for AsB and DMA in BCR-627 reference material are 3.90 ± 0.22 and 0.15 ± 0.02 mg kg^{-1}, respectively.
[e] Non-detectable.
[f] Detection limits for AsB, As(III), DMA, MMA, and As(V) are 0.022, 0.015, 0.016, 0.014 and 0.017 mg kg^{-1} in fish tissues.

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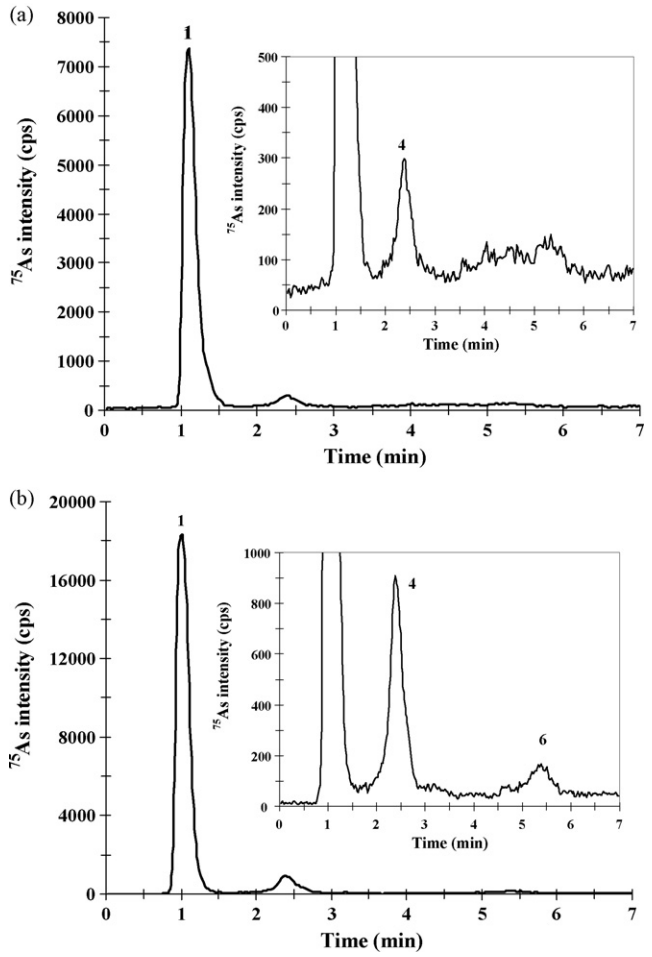

Fig. 3. IC–ICP–MS profile of enzymatic extracts of (a) BCR-627 and (b) DOLT-3 obtained by MAEE. Peak identification: (1) AsBet, (4) DMA, and (6) As(V).

low total As extraction efficiency in this reference material can be partially accounted for by the nature of fish tissue (fibrous with a significant range of particle size) [13].

The method was applied to the analysis of commercial fish tissues showing quantitative recoveries of total As for canned tuna and shark; lower As extraction recovery was obtained for marlin tissue. The percentage of total As extracted in marlin was 63 ± 1%. The lower recovery was due to the difficulty of obtaining complete extraction in this fish tissue. It has been reported that the presence of other compounds, such as lipids, can reduce the extraction efficiency of As species in fish tissues [12].

IC–ICP–MS chromatograms of enzymatic extracts of BCR-627 and DOLT-3 obtained by MAEE under the optimized conditions are shown in Fig. 3. Speciation recoveries were calculated by comparing the sum of As species concentrations with the total As extracted. The results of species quantification are shown in Table 4. Results of total As in the extracts and the sum of each As species quantified were in good agreement.

As speciation results were validated by the analysis of BCR-627, which is certified for AsB and DMA (Fig. 3a). No significant differences were found between the certified values and the results obtained by using MAEE during this study at the 95% confidence level (Table 4). In DOLT-3 reference material (Fig. 3b), the major species was AsB (86%), while the levels of DMA and As(V) were much lower. Different relative As distribution among these species was reported in DOLT-2 by Wahlen et al. [13]. The two major species in DOLT-2 tissue extracted by sonication with water were AsB (67%) and DMA (14%). It can be seen from Table 4 that AsB was found to be the predominant As species in the commercial fish samples. The amount of As determined in fish tissues varied depending on the type of fish analyzed, but the percentage of AsB was always higher than 87% of the total As extracted. Considerable concentrations of As(III) in shark tissue were detected. The levels of DMA, MMA and As(V) were much lower in the analyzed fish tissues.

Table 5
Analytical results for total Se and SeMet after the proposed MAEE procedure. The results are indicated at 95% CL for $n = 3$.

Sample	Total Se extracted (mg kg^{-1})	SeMet (mg Se kg^{-1})[c]
BCR-627 (IRMM, Belgium)	1.6 ± 0.2 (57 ± 9)[a]	0.6 ± 0.1 (38 ± 6)
DOLT-3 (NRCC, Canada)	5.9 ± 0.2 (84 ± 3)[b]	0.5 ± 0.1 (9 ± 2)
Canned tuna (in water)	5.5 ± 0.2 (98 ± 3)[a]	1.6 ± 0.1 (29 ± 2)
Shark	0.9 ± 0.1 (46 ± 5)[a]	0.5 ± 0.1 (56 ± 11)
Marlin	3.5 ± 0.1 (89 ± 3)[a]	1.6 ± 0.1 (46 ± 3)

[a] In parentheses, the extraction recovery based on the total Se determined by microwave-assisted total digestion (EPA Method 3052).
[b] In parentheses, the extraction recovery based on total Se certified value.
[c] In parentheses, SeMet recovery based on total Se extracted.

3.6. Determination of total Se and Se species in fish tissue extracts after MAEE procedure

As can be seen from Table 5, the percentages of extractable Se for the certified reference materials were 57 ± 9% for BCR-627 and 84 ± 3% for DOLT-3. Díaz Huerta et al. [20] reported total Se recoveries of 63% when conventional enzymatic hydrolysis using protease XIV/lipase was applied to cod muscle BCR-422 reference material. In the commercial fish tissues, the Se recoveries ranged from 46% (shark) to 98% (canned tuna). The extraction recoveries of Se were comparable with other reported data in fish and shellfish samples after enzymatic extraction with proteolytic enzymes. The extraction recoveries were between 65 and 96% [5,21].

It is interesting to observe that the sum of Se species detected by IC–ICP–MS was much lower than the total Se in the resulting enzymatic extracts of all fish tissues studied. SeMet was the only Se compound detected in the samples. Fig. 4a shows a chromatogram of BCR-627 extract after MAEE. Results for speciation analysis in fish tissue extracts are shown in Table 5. In the reference materials BCR-627 and DOLT-3, 38 and 9%, respectively, of the total Se extracted was identified as SeMet. For other analyzed fish tissues, the total amount of SeMet varied depending on type of fish. In shark, 56% of the total Se extracted was SeMet, whereas canned tuna presented lower percentage of SeMet (29% of the total Se extracted). Percentages of SeMet for these fish species were comparable with other reported values in fish tissues between 28 and 93% for tuna and swordfish, respectively [5] and shellfish (35% for Antarctic krill) [10] after enzymatic hydrolysis. It has been demonstrated that during the enzymatic extraction of marine tissues, some Se compounds might remain bound in peptide form, depending on the cleavage specificity of the enzyme and the analyzed fish species [5,6,10,21]. In order to confirm those results, enzymatic extracts of fish tissues were analyzed by SEC–ICP–MS. The chromatographic conditions are listed in Table 1. The chromatographic profile for BCR-627 is shown in Fig. 4b. Two fractions of high molecular weight (fractions II and III) with mass ranges (Mr) between 20 and 200 kDa, and a fraction of low molecular weight (fraction I, 20 ≥ Mr ≥ 0.2 kDa) were clearly differentiated after molecular mass calibration of the column (using transferrin, albumin, myoglobin, insulin and SeMet). The low molecular mass range (fraction I) showed the match in retention time of a peak at 8.2 min and SeMet standard, which represented approximately 40% of the total Se in the extract. The results obtained for BCR-627 by SEC were in agreement with the quantitative data of SeMet obtained by IC.

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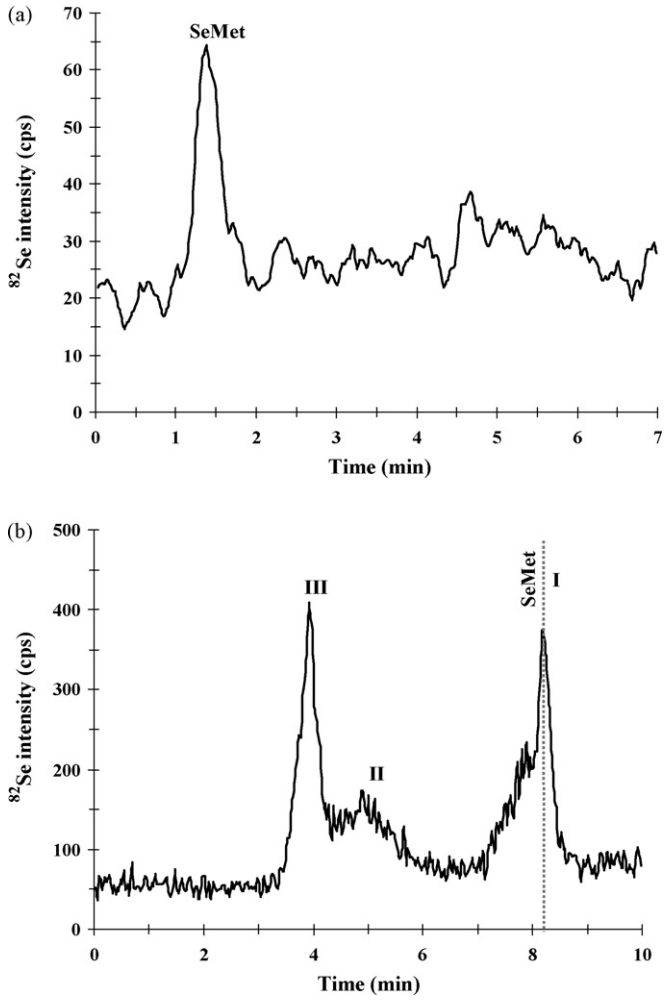

Fig. 4. Typical chromatograms obtained by the analysis of BCR-627 extract from MAEE using: (a) anion-exchange chromatography (IC) and (b) size exclusion chromatography (SEC). For Se chromatogram (b), identified fractions were I (20–0.2 kDa), II (150–20 kDa) and III (>150 kDa).

4. Conclusions

The results presented in this study demonstrate for the first time the potential of microwave technology to assist enzymatic extraction of As and Se species from fish tissues prior to their analysis by IC–ICP–MS. MAEE using pronase E/lipase mixture provided, in most of the cases, good extraction efficiencies without chemical alteration of the As and Se species during the extraction process. Additionally, a significant reduction in the extraction time (30 min) was attained using closed vessel MAEE, compared with conventional enzymatic extraction (20 h), simplifying sample preparation steps. Extraction recoveries were in the range of 63 ± 1 to $106 \pm 2\%$ for As and 46 ± 5 to $98 \pm 3\%$ for Se.

For As species, good agreement was obtained between the sum of the species quantified and the total As found in the extracts. In all the fish tissues investigated, the As existed almost exclusively in the form of AsB. For Se, SeMet was the only Se compound found in fish tissues after the enzymatic hydrolysis process. Moreover, SeMet content corresponds to between 29 and 55% of the total Se extracted during the enzymatic extraction since some Se-peptides or Se-containing proteins remained intact.

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